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The applicability of fluorescence lifetime to determine the time since deposition of biological stains

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Age estimation of bloodstains is of importance to a forensic investigation in that it gives an indication as to when the crime was committed and can discern whether the bloodstains resulted from a single event or were deposited on different occasions. To date, there is no established method for determining the age of bloodstains and therefore this work investigates the potential of using fluorescence lifetime measurements to determine the time since deposition of bloodstains. Experiments were carried out using human blood obtained from 6 donors. The results showed a decrease in fluorescence lifetime within the first 91 hours of deposition, after which the fluorescence lifetime plateaued, illustrating a clear distinction between fresh and old bloodstains. The results also show the applicability of this method to stains originated from a blood/saliva mix (5:1 ratio). Saliva stains aged with the same parameters and conditions as blood showed no change in fluorescence lifetime, suggesting that this phenomenon only takes place in blood and not saliva. Further analysis on test stains revealed that measuring the fluorescence lifetime over a period of time provides information on their freshness and can therefore form the basis of a valuable bloodstain dating technique.

Introduction

Bloodstains, which are commonly encountered at crime scenes, are forensically valuable since they may provide DNA profiles which can be used to identify a victim or suspect, whilst blood pattern analysis can be used to reconstruct the crime.^{1, 2} Knowing the time at which a bloodstain was deposited is also beneficial as it can assist forensic investigators in ascertaining when the crime was committed and whether the bloodstains resulted from a single event or multiple events.^{2, 3} This information can also save the police time and resources if the age of the stain failed to coincide with the time the crime was known to be committed.³ It would therefore be very helpful if a technique could be developed that could reliably determine the age of a bloodstain, and this has been addressed by several researchers in the field.²⁻⁴

The methods that have been proposed to estimate the age of bloodstains do so by exploiting the physical, chemical, biological and molecular properties of blood (Table 1), with a substantial number of these approaches (oxygen electrode method,⁵ reflectance spectroscopy,⁶ and electron paramagnetic resonance⁷) based on the transformation of haemoglobin into its degradation products, methaemoglobin and hemichrome.^{2, 4} This oxidative process in blood is accompanied by a colour change from red to brown, and this

too has been used to determine the age of bloodstains, dating back to the early 1900's.² Other methods, which do not rely solely on these physical changes in blood, include high performance liquid chromatography (HPLC),⁸ entomological estimation⁹ and RNA analysis.¹⁰ While these approaches have been shown to estimate the age of bloodstains, a number of drawbacks have been associated with them, including susceptibility to environmental conditions (e.g. storage temperature, light exposure and humidity), causing the bloodstain to appear fresher or older than it really is and resulting in errors in the estimation of the age of the stain.^{5, 7, 8, 10} Moreover, to prevent samples from clotting soon after being drawn, most experimental techniques have used blood that has been treated with anti-coagulants such as EDTA or heparin.^{2, 5, 8, 10} This is problematic because the stains encountered in real-case scenarios will be untreated and therefore the age obtained with the use of anti-coagulants in a laboratory setting may not be representative of the actual age of a bloodstain. Furthermore, a few of these procedures have shown variation in age estimation depending on the type of surface the blood was deposited on.¹¹ In general, most of these proposed techniques have been associated with high running costs, are time-consuming, cumbersome and may require highly trained personnel, thus making them economically unfeasible.

In light of all these challenges, a novel technique for determining the age of a bloodstain using the fluorescence lifetime of tryptophan emerged in 2012.^{12, 13} Tryptophan, which is one of the few aromatic amino acids and the dominant endogenous fluorophore in proteins, was selected because it is a component of the two major blood proteins,

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Table 1. Summary of techniques proposed for age determination of deposited bloodstains.

Method	Description	Ref
Oxygen electrode	Measures the physico-chemical changes that occur in haemoglobin upon leaving the body (haemoglobin → methaemoglobin → hemichrome). While these techniques were able to estimate the age of stains, they were affected by changes in environmental conditions such as temperature, light exposure and humidity.	5-7
Reflectance spectroscopy		
Electron paramagnetic resonance		
High performance liquid chromatography (HPLC)	Detects a unique peak "X" in blood, suspected to be a polar, low molecular weight denaturation product. This peak was absent in recently drawn blood, but its area increased as the freshness of the blood decreased. While HPLC was proven to be highly sensitive for this purpose, the effect of unusual storage conditions often encountered in forensic casework may complicate data interpretation.	8
Entomological estimation	Uses larvae found on deposited bloodstains to estimate the age of the bloodstain in a way similar to determining the Post Mortem Interval of a corpse. Nevertheless, this method assumes that oviposition occurs soon after the crime was committed which may not necessarily be true and can underestimate the age of the stain.	9
RNA Analysis	Measures the ratio of 18s RNA to β -actin mRNA over time. However, the major concern surrounding this method is the sensitivity of nucleic acids to sunlight, humidity, temperature and microbial activity, as this can accelerate RNA degradation and lead to the over-estimation of the age of a stain.	10
Fluorescence lifetime	Measures the fluorescence lifetime of tryptophan; the dominant endogenous fluorophore of blood proteins. Fluorescence lifetime is independent of concentration, allowing for trace amounts of blood to be analyzed, is quick, and uses untreated whole blood which is representative of stains encountered at crime scenes.	12, 13

albumin and gamma globulin and is also very sensitive to its environment.¹²

Fluorescence lifetime, which is characteristic of all fluorophores, reflects the time a fluorophore stays in the excited state before it re-emits light.¹⁴ Based on experiments conducted with canine blood, Guo *et al.* hypothesized that, as a bloodstain ages, structural changes occur within the major blood proteins (e.g. albumin and gamma globulin), that reduce the fluorescence lifetime of tryptophan allowing for age estimation within the first week since deposition.¹² A key feature of fluorescence lifetime is that it is independent of fluorophore concentration,^{15, 16} which means that the age of

microscopic bloodstains can still be determined. Likewise, different-sized stains, deposited at the same time, will have similar fluorescence lifetime values. Some aspects of this approach are similar to radiocarbon dating, which rely on determining the amount of radioactive ¹³C in organic matter, albeit over much larger time scales.

In this present study, the use of fluorescence lifetime measurements in determining the time since deposition of a bloodstain was further explored, with the scope expanded to include another body fluid (saliva) and mixtures of blood and saliva. Human donors were also used and fluorescence lifetime measurements were taken at an excitation wavelength of 281nm (to account for both Trp and Tyr contributions). The

applicability of fluorescence lifetime to forensic casework was also assessed using simulated test stains. From the data, a fluorescence decay model was developed and evaluated to predict the age of bloodstains. Moreover, a further means to confirm the correctness of the prediction has been suggested. Overall, this signifies a more comprehensive analysis of fluorescence lifetime measurements and it includes aspects not fully examined prior to this which confirm the potential of the technique and the need for it to be considered along with other prospective methods for bloodstain age estimation.

Materials and methods

Sample collection and preparation

All the experiments were performed in compliance the UK law and the King's College London (KCL) institutional guidelines. Ethical approval for sample collection was granted by the KCL Research Ethics Committee (BDM/12/13-101). Informed consent was obtained for all sample collections.

Venous blood (20 mL) was drawn from six healthy human donors (after receiving informed consent) and decanted into 50 mL Falcon tubes free from anti-coagulants. Immediately after drawing, 3 mL of blood from each volunteer was plated in triplicate on to plastic petri-dishes.

Saliva (5 mL) from each donor was collected in 10 mL sample tubes at the same time the blood samples were taken. Using the same donors, bloodstains mixed with saliva were made by plating 2.5 mL of blood in triplicate, and mixing these samples with 0.5 mL of saliva to give a blood-saliva ratio of 5:1.

Pure saliva (3 mL) was plated separately in the same manner as blood. The blood, blood mixed with saliva and saliva stains were left uncovered in the laboratory and exposed to room temperature ($23 \pm 2^\circ\text{C}$), a humidity of $40 \pm 5\%$ and ambient light.

At daily intervals for approximately 1200 hours, the three tests (blood, blood mixed with saliva and pure saliva) were sampled by removing a scraping of the dried blood from the centre of each petri dish using a spatula. This was transferred to 1.5 mL microfuge tubes containing 1 mL of phosphate buffer saline (Oxoid Ltd, Basingstoke Hampshire, UK). Efforts were made to standardise sampling by scraping the centre of each plate twice, however, since fluorescence lifetime is independent of concentration, any variation in sampling would not affect the fluorescence lifetime measurement. The samples were then conducted on pure bloodstains, bloodstains mixed with saliva, saliva stains and control stains to investigate the fluorescence lifetime changes at each time point. A paired t-test between the fluorescence lifetime values for pure bloodstains and bloodstains mixed with saliva was also conducted to assess whether the presence of saliva affected fluorescence lifetime.

Time since deposition determination

To assess the ability of the fluorescence lifetime method to predict the age of a bloodstain, test stains from 4 human donors were prepared. After a period of 21 hours, the centre

vortexed and sonicated for 1 minute. Undissolved material was removed by centrifugation (10g; 1 minute). The supernatants were then transferred to fresh microfuge tubes and were subsequently used for fluorescence lifetime measurements.

For the control sample preparation, 36 mg of L-tryptophan, bovine gamma globulin and bovine serum albumin (Sigma-Aldrich Company Ltd, Dorset, England) were each dissolved in 9 mL of PBS buffer. Each protein preparation (3 mL) was then plated onto plastic petri-dishes in triplicate and treated in the same manner as the other samples.

Fluorescence lifetime measurements

The fluorescence lifetime of each sample was measured with a FluoroCube-HORIBA fluorescence lifetime spectrofluorometer (Horiba Jobin Yvon, Stanmore Middlesex, UK) using the time correlated single photon counting (TCSPC) technique. A 280 nm pulsed diode light source NanoLED[®] with a peak wavelength of 281 nm and an optical pulse duration of < 1.0 ns was used for excitation, at a repetition rate of 1 MHz (Horiba Jobin Yvon, Stanmore Middlesex, UK).¹⁷ The slit on the monochromator was set at 32 nm. The emission wavelength was set at 330 nm while the peak pre-set was set to 1000 counts. An IBH model TBX-04 photon detector was used (Horiba Jobin Yvon, Stanmore Middlesex, UK). Ludox diluted in distilled water (1 in 1000) was used to measure the instrument response function at an excitation and emission wavelength of 281 nm before any measurements was taken. Each sample (100 μL) was then added to a quartz cuvette (Hellma[®] Analytics, Germany) and topped up to 2 mL with PBS.

Fluorescence lifetime data analysis

The decay analysis software DAS6 (Horiba Jobin Yvon, Stanmore Middlesex, UK) was used to perform a two-exponential fit of all the samples. The average lifetime (T) was calculated from the two components T_1 and T_2 and the fractional contribution (B) using the following equation:

$$\text{Average fluorescence lifetime, } T = T_1B_1 + T_2B_2$$

The average fluorescence lifetimes of blood, blood mixed with saliva, pure saliva, L-tryptophan, bovine gamma globulin and bovine serum albumin were then plotted as a function of time, and fitted with a bi-exponential decay function to yield a calibration curve of average fluorescence lifetime versus time. Repeated measures of ANOVA and paired t-tests were

of each plate was scraped with a spatula and the blood sample transferred to a tube containing 1 mL PBS. For each donor, this was carried out in triplicate and treated in the same manner as detailed above. The fluorescence lifetime value of each sample was then compared to the previously constructed calibration curve of fluorescence lifetime versus time, and its age determined. This procedure was then repeated after 44 hours, 140 hours and 524 hours. To determine whether consecutive fluorescence lifetime measurements can provide additional information on the age of the stain, test stains were set up using 3 mL of venous blood from two human donors. After 21

hours, the fluorescence lifetime values of the stains were measured and recorded as “Day 1”. On Day 2, when the stains were 45 hours old (21 hours + 1 day) and on Day 3 when the stains were 69 hours old (45 hours + 1 day), the fluorescence lifetime values were again measured and recorded.

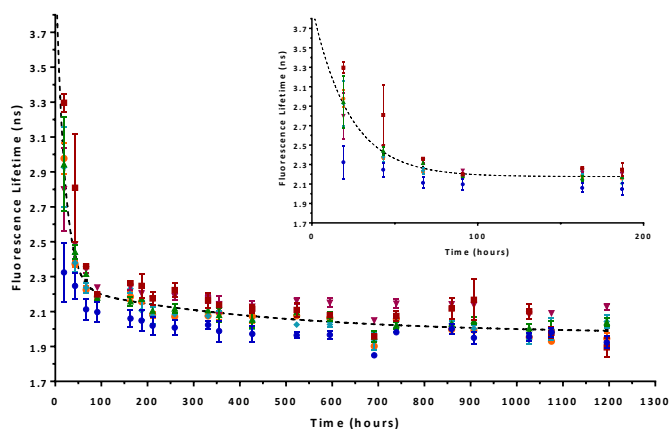


Figure 1. Decrease of the average fluorescence lifetime for blood aged up to 1200 hours; ($n=6$). The trend line, calculated from a bi-exponential fit, is representative of the median fluorescence lifetime of the six donors at each time point. Precision of fluorescence lifetime measurements for each time point is shown by the standard error of mean (SEM) using the average of three replicate samples taken from each donor. Insert shows data for the first 190 hours after plating.

The blood stains were then aged for a further 75 hours so that they were 144 hours old (Day 1), and their fluorescence lifetime values measured. Fluorescence lifetime measurements were also taken when the stains were 168 hours old (Day 2) and 192 hours old (Day 3).

Results and Discussion

Fluorescence decay of bloodstains

The fluorescence lifetime measurements of blood taken from 6 donors over a period of 1200 hours are presented in Figure 1. The fluorescence lifetime of blood was measured at an excitation wavelength of 281nm, aimed at exciting endogenous fluorophores present in blood proteins.^{16, 17} These included tyrosine and tryptophan residues as their fluorescence lifetime changes can both be exploited to probe potential structural rearrangements of proteins (e.g. albumin) taking place during the aging process.¹⁸ Amplitude weighted mean fluorescence lifetimes were used and the results showed that the fluorescence lifetime of human blood decreased non-linearly over time from ~ 3.0 ns, 19 hours after deposition, to ~ 2.0 ns after 50 days of aging (Figure 1). This was also demonstrated when the fluorescence decay of a representative bloodstain after 19 hours of deposition was compared to the decay of the same bloodstain after 50 days of deposition (Figure 2a). As illustrated, the slope of the decay curve decreased over the 50-day period and thus the fluorescence lifetime was reduced. However, statistical analysis indicated that this decrease was most significant within the first 91 hours of the blood being deposited ($p <$

0.05), after which there was little variation in the fluorescence lifetime values ($p > 0.05$) (Figure 1 insert). For comparison, the same analysis was conducted by replacing amplitude weighted- with intensity weighted mean fluorescence lifetimes

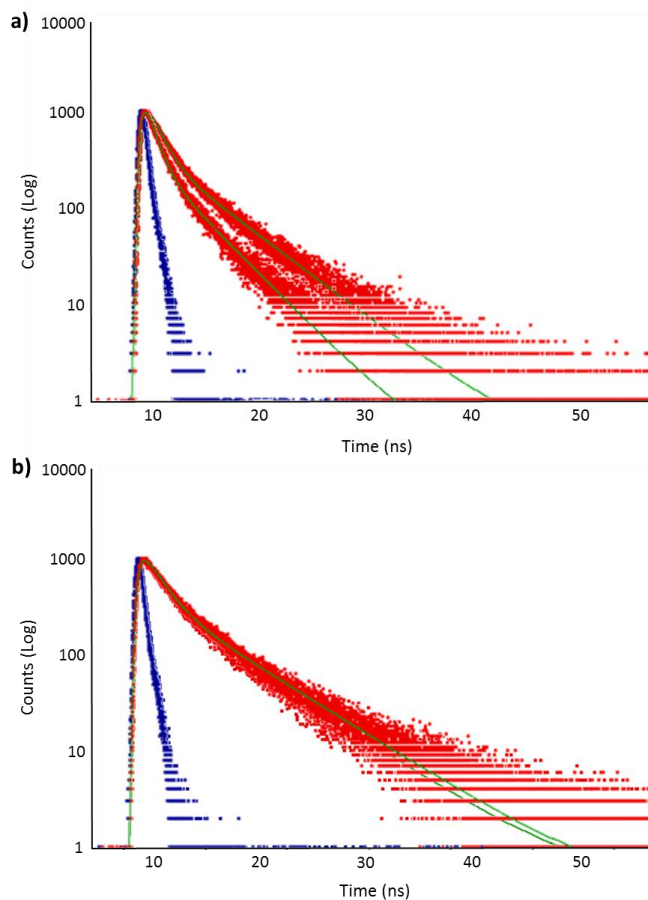


Figure 2. Selected decays of a bloodstain (a) and saliva stain (b) after 19 hours of deposition (top line) and 50 days of deposition (lowest line).

¹⁸ (data not shown) with similar results being obtained in both cases.

The data analysis also revealed a certain degree of inter-donor variation (Figure 1). Such variability would need to be considered, particularly if precise age estimation was attempted. Despite this, there was a consistent trend observed in all donors where the fluorescence lifetime decreased non-linearly within the first 91 hours of deposition. This proves that fluorescence lifetime changes with age, and demonstrates the potential to use these measurements as a method for determining the freshness of deposited bloodstains.

Fluorescence decay of bloodstains mixed with saliva

Mixtures of biological fluids are common in forensic science, and for example, bloodstains mixed with saliva can often appear at crime scenes where there is a wound to the mouth, throat or chest.¹⁹ For age determination of bloodstains, it is therefore important to evaluate any effect that another body

fluid may have on the measurement. Previously reported techniques have not investigated mixtures,^{5-8, 10} but it is important that this is taken into account. As with pure bloodstains, bloodstains mixed with saliva experienced a sharp decrease in fluorescence lifetime within 91 hours of being deposited ($p < 0.05$) (Figure 3). This was also illustrated in

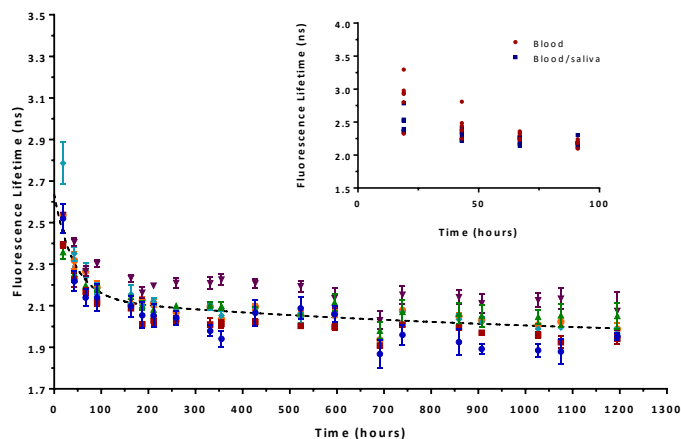


Figure 3. Decrease of the average fluorescence lifetime of blood mixed with saliva aged up to 1200 hours; ($n=6$). The trend line, calculated from a bi-exponential fit, is representative of the median fluorescence lifetime of the six donors at each time point. Precision of fluorescence lifetime measurements for each time point is shown by the standard error of mean (SEM) using the average of three replicate samples taken from each donor. Insert shows data for the first 91 hours after plating.

Figure 4, where the fluorescent lifetime values of pure bloodstains and bloodstains mixed with saliva less than 91 hours old can be easily distinguished from those that are older than 91 hours. Further statistical analysis revealed that there was no significant difference between the fluorescence lifetime of blood and blood mixed with saliva ($p > 0.05$) and this is reflected in the overlap of data points for both blood and blood mixed with saliva (Figure 3; insert). Nevertheless, it is worth noting that these results are only reflective of a 5:1 blood/saliva ratio and therefore to determine the effect of different amounts of saliva on the fluorescence lifetime of blood, a wider validation study will need to be conducted.

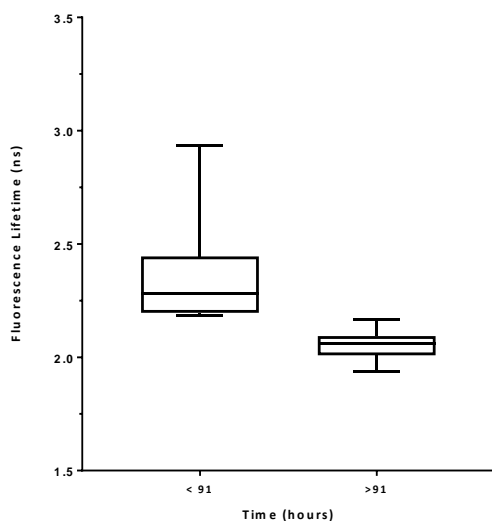


Figure 4. Box plot showing the fluorescence lifetime values for both pure bloodstains and bloodstains mixed with saliva that are less than 91 hours old and more than 91 hours old.

Fluorescence decay of saliva stains

Saliva is one of the most common body fluids found at a scene of crime²⁰ and as such, the application of fluorescence lifetime measurements in the age determination of bloodstains was extended to saliva stains, to investigate whether saliva performs in a similar way to blood.

Furthermore, salivary amylase (the major protein constituent of saliva) contains aromatic amino acid residues^{21, 22} making it a suitable candidate for fluorescence lifetime measurements. Saliva stains were obtained from the same blood donors and measured over 1200 hours. Figure 5, which represents the average fluorescence lifetimes of saliva, showed that the fluorescence lifetime of pure saliva (~3-4 ns) was longer than the lifetime of the other samples, which ranged between 3-2 ns. This was to be expected since the amino acid composition and the protein conformation of amylase differ from that of albumin or gamma globulin, and as such, the fluorescence lifetime value of each of these proteins will be distinct.²³ It was also observed that the lifetime values of saliva remained unchanged for the entire aging process which was confirmed by statistical analysis ($p > 0.05$). This was also shown in Figure 2b where the slope of the decay curve after 19 hours of deposition did not differ significantly from the slope of the decay curve after 50 days.

Overall, these results suggest that although fluorescence lifetime measurements are applicable to blood, the technique is not suitable for the age estimation of saliva stains.

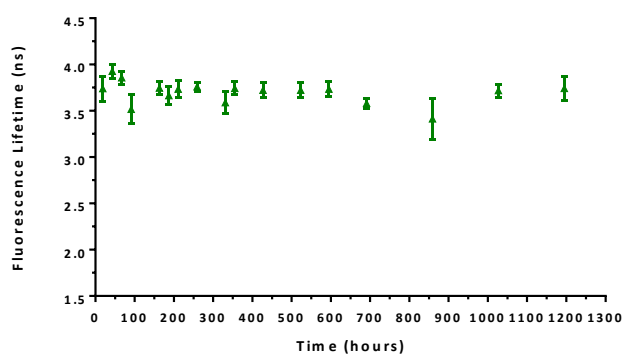


Figure 5. The average fluorescence lifetimes of saliva over a period of 1200 hours. Each time point represents the average fluorescence lifetime of six donors. Precision of fluorescence lifetime measurements for each time point is shown by the standard error of mean (SEM).

Fluorescence decay of control samples: L- tryptophan, bovine gamma globulin and bovine serum albumin

Control experiments were carried out to investigate whether tryptophan free in solution and tryptophan/tyrosine containing proteins (bovine gamma globulin and bovine serum

albumin) would produce similar results to those obtained with blood. As illustrated in Figure 6, free tryptophan was found to have an average fluorescence lifetime of 2.6 ns which is comparable to the values reported in literature.^{12, 24} Bovine serum albumin and bovine gamma globulin which has more

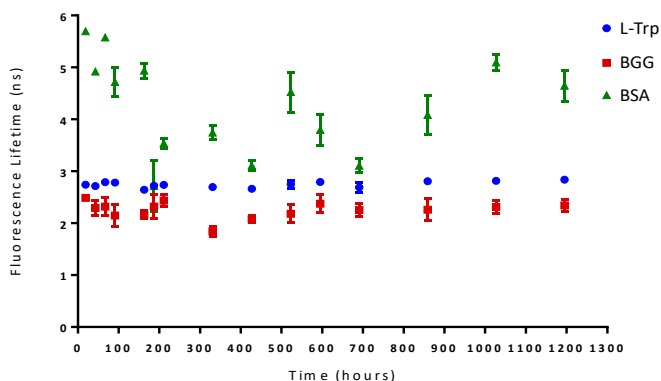


Figure 6. Average fluorescence lifetimes of control samples L-tryptophan, bovine gamma globulin and bovine serum albumin over a period of 1200 hours. Precision of fluorescence lifetime measurements for each time point

than 10 tryptophan residues¹⁴ had a fluorescence lifetime of 4.3 ns and 2.3 ns respectively.

Although bovine gamma globulin had a similar lifetime to tryptophan free in solution, the fluorescence lifetime of bovine serum albumin was observed to be almost double that of bovine gamma globulin, providing further evidence that the environment strongly influences the fluorescence lifetime of the intrinsic fluorophores.

Despite all samples being exposed to the same external conditions, it was observed that the lifetimes of L-tryptophan, bovine serum albumin and bovine gamma globulin remained relatively constant over time ($p > 0.05$) while the fluorescence lifetime of blood decreased drastically in the first 91 hours. This suggests that changes within the microenvironment of blood potentially associated with the blood aging process may be responsible for the observed decrease in lifetime, rather than environmental factors.

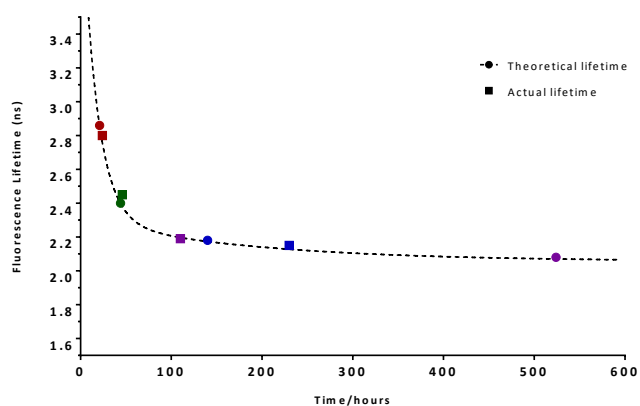


Figure 7. Fluorescence lifetime versus time showing the theoretical fluorescence lifetime values (circles) that correspond to the true ages of the four test stains (extrapolated from Figure 1) compared to the actual fluorescence lifetime values and ages obtained for these stains.

Time since deposition determination of bloodstains

To further confirm the applicability of the technique to predict the age of bloodstains, blood from a new set of donors was deposited and aged for a total of 524 hours with samples collected and analysed at different time points (21 hours, 44 hours, 140 hours and 524 hours).

The fluorescence lifetime values obtained were then used to determine the ages of stains based on the calibration of average fluorescence lifetime versus time, shown in Figure 1.

As illustrated in Figure 7, the prediction was reliable for bloodstains aged 21 hours and 44 hours, but it failed for older stains, in agreement with the results from the statistical analysis which showed a significant change in fluorescence lifetime only within 91 hours of deposition.

It was also investigated whether measuring the fluorescence lifetime of a bloodstain over time can provide additional information regarding the age of the stain. Blood from donors was deposited and the fluorescence lifetime of each stain was measured over a three-day period (at time points: 21 hours and 144 hours). As illustrated in Table 2, the stains aged for 21 hours showed a continuous decrease in fluorescence lifetime over the three-day period which coincides with the region of the fluorescence decay curve where a drastic decrease in lifetime is observed for stains that are ≤ 91 hours old.

Table 2. Fluorescence lifetime values of two stains measured over a three-day period at two time intervals, $t = 21$ hours and $t = 144$ hours.

	Age of bloodstain (hour)	Fluorescence Lifetime (ns)		
		Day 1	Day 2	Day 3
S1	21	3.02	2.69	2.59
	144	2.50	2.49	2.44
S2	21	2.90	2.83	2.62
	144	2.54	2.53	2.52

However, the stains aged up to 144 hours showed small changes in the fluorescence lifetime, which coincides with the region of the fluorescence decay curve where the stains were more than 91 hours old. This information can be very useful to forensic investigators who would be able to differentiate between a freshly deposited stain and an older stain. Such a test would have the advantage of being completely unaffected by interindividual variability and therefore represents a means to further confirm the correctness of a prediction.

Conclusions

This research indicates that fluorescence lifetime measurements can be used to distinguish fresh stains from very old ones. Fluorescence lifetime is also applicable to bloodstains mixed with saliva, but was proven to be unsuitable for dating pure saliva stains. Overall, the technique required little sample preparation and was found to be reproducible, quick and simple making it ideal for forensic casework. Furthermore, fluorescence lifetime is independent of

fluorophore concentration allowing for the analysis of very small bloodstains that may be encountered at crime scenes. Whilst the exact molecular mechanism of blood aging is unknown, Guo *et al.*¹² suggested that as a bloodstain ages, structural changes can occur in blood proteins, and since tryptophan is sensitive to its environment, the changes can affect the fluorescence lifetime. It is conceivable that protein degradation and/or digestion by enzymes upon deposition and exposure to the environment may play a role in the observed decrease in fluorescence lifetime. It is also possible that other blood proteins, such as fibrinogen and regulatory proteins can be contributing to the fluorescence lifetime measurement obtained for blood, as these are composed of both tyrosine and tryptophan residues that can be excited at 280nm. To fully understand why the fluorescence lifetime of blood decreases over time and what is responsible for this decrease, future studies will investigate how the fluorescence lifetime of blood varies with different environmental conditions as well as the role of proteins and endogenous fluorophores in the blood aging process.

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